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STUDIES ON SERUM B_{12} ASSAY

with Special Reference to Its Use in the Diagnosis of Vitamin B_{12} Deficiency

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Studies on Serum B_{12} Assay

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The present paper comprises a summary of earlier investigations, which have been published in the following articles.

I. The assay of vitamin B_{12} in human serum.
Acta Soc. Med. Upsal. 62, 39 (1957).

II. The serum vitamin B_{12} levels at various ages.
Acta Paediat. 46, 585 (1957).

III. The use of the serum vitamin B_{12} assay in the diagnosis of vitamin B_{12} deficiency.
Acta Med. Scand. 159, 307 (1957).

IV. Subacute combined degeneration of the spinal cord. The diagnostic value of serum vitamin B_{12} assay.
Acta Med. Scand. 160, 75 (1958).

V. Megaloblastic anaemia associated with pregnancy or puerperium. Report of three cases with normal serum vitamin B_{12} levels and a subsequent response to treatment with vitamin B_{12}.
Acta Haemat. 19, 9 (1958).

VI. Oral treatment of pernicious anaemia with vitamin B_{12} and purified intrinsic factor. I. The value of serial estimation of the vitamin B_{12} levels of serum.
Acta Med. Scand. 160, 339 (1958).

VII. Oral treatment of pernicious anaemia with vitamin B_{12} and purified intrinsic factor. II. Studies on the reduced effect of prolonged treatment.
Acta Soc. Med. Upsal. 63, 1 (1958).

In the following these articles will be referred to using the Roman numerals given above.

Introduction

Before the introduction of the microbiological assay of vitamin B_{12} in serum, deficiency of the vitamin could be diagnosed only on the appearance of typical symptoms and signs and on the occurrence of haematological response following therapy with liver extract or crystalline vitamin B_{12}. Ross (11) was the first to demonstrate lower values for the serum B_{12} in pernicious anaemia than in normal subjects. In 1952 he gave a detailed description of the assay of vitamin B_{12} in body fluids using *Euglena gracilis* var. *bacillaris* (12). At the same time Mollin and Ross (10) confirmed Ross' original observation on a larger series of patients with pernicious anaemia. In subsequent years similar results have been reported by several investigators using different techniques of assay.

The information in the literature, however, on the serum B_{12} concentration in normal subjects and in patients with various diseases differs widely. These differences cannot be attributed solely to the use of various test organisms, since the results obtained by investigators using the same organism vary as greatly as those found when different test organisms have been employed (I). It is not known to what extent the differences are due to the use of varying techniques of assay or to dissimilarities in the composition of the series investigated. Thus rigidly controlled and standardized assay conditions are needed, and each investigator in this field must define normal and pathological serum B_{12} values under his own conditions of assay.

Plan of Investigation

The investigations to be presented were at first devoted to the method with special reference to its accuracy (I). Then the serum B_{12} levels of haematologically normal subjects at all ages were determined (II). The use of serum B_{12} assay in the diagnosis of various disorders, mainly haematological, was also investigated (III). Special attention was paid to the diagnosis of glossitis (III) and subacute combined degeneration of the spinal cord (IV) in patients with slight or no anaemia. The findings in patients with megaloblastic anaemia associated with pregnancy or puerperium differed from those obtained in other megaloblastic anaemias and were discussed separately (V). The method was also used to control the effect of oral maintenance therapy in pernicious anaemia (VI). Finally, certain problems were investigated concerning the reduced effect of prolonged oral maintenance treatment (VII). In this investigation, both serum B_{12} assays and absorption tests with radioactive vitamin B_{12} were employed.

The investigations were performed from 1953 to 1957. During the course of the work it was found that patients with chronic myelocytic leukaemia had very high serum B_{12} concentrations (7). Moderately elevated to normal serum B_{12} levels were observed in patients with acute leukaemia, polycythaemia vera, liver cirrhosis and diabetes mellitus. Patients wih these diseases have been omitted in the investigations under discussion, except those who suffered from both pernicious anaemia and diabetes mellitus.

Method

The small vitamin B_{12} content of blood serum necessitates the use of microbiological methods of assay. Of the various test organisms available, *Euglena gracilis* seems to be the most suitable with regard to sensitivity as well as to specificity. Three strains of *Euglena gracilis* are at present employed: var. *bacillaris, strain T* and *strain z*. The first one discovered (*Euglena gracilis* var. *bacillaris*) has been used in almost all investigations published up to now and was used in this laboratory until November 1956. Since then, *Euglena gracilis strain z* (6) has been employed. Compared to the *bacillaris* strain, it grows more rapidly and is more sensitive. No systematic differences were noted between the results obtained with the two test organisms in parallel assays, which is in agreement with the experiences of Ross *et al.* (13).

The microbiological assay using *Euglena gracilis* var. *bacillaris* is described in the first paper (1). The principle is as follows. The growth-promoting effect of dilutions of serum is compared with that of solutions with known concentrations of crystalline vitamin B_{12}. The vitamin B_{12} activity of the sample is expressed as the serum B_{12} concentration in $\mu\mu$g per ml. Ross' method was followed with some modifications. After inoculation with a washed and diluted culture and subsequent incubation for 10 days, the growth of *Euglena* was estimated by photometric determination of the pigment, extracted from the cells with concentrated ethanol.

The accuracy of the method was studied in three ways. Recovery tests were performed by adding 50–400 $\mu\mu$g of vitamin B_{12} to different sera. On subsequent assays the recovery ranged from 83 to 119 per cent. The variation between different assays was studied by determinations of the same sample in 14 consecutive assays. The mean serum B_{12} concentration was 400 $\mu\mu$g per ml and the standard deviation 54 $\mu\mu$g per ml. Double determinations in different assays were performed on 243 sera. The mean value of the differences calculated in per cent of the average of the two determinations was found to be – 1.1 per cent. The frequency distribution of the differences was given and

the standard deviation calculated as 21.3 per cent. The conclusion arrived at is that though the method is very sensitive there is a considerable degree of variation.

The Serum B_{12} Levels at Various Ages

In contrast to the numerous reports in the literature on the serum B_{12} concentrations in various diseases, little attention has been paid to the serum levels of the vitamin in health at various ages. In this investigation, the serum B_{12} levels were determined in 242 haematologically normal subjects of both sexes at various ages from birth to 82 years (II).

The range of the serum B_{12} values was found to be wide in both sexes at all ages. The serum B_{12} levels in newborn infants were significantly higher than those in the mothers. The latter had less vitamin B_{12} in the serum than non-pregnant women of the same age. The biological significance of these findings is not known, but it may be recalled that the cord blood contains also a higher content of other substances, e.g. serum iron and amino acids. It is not known whether and if so to what extent the lower serum B_{12} levels in late pregnancy are due to simple haemodilution. Obviously, a foetal demand exceeding the ability of the mother to absorb the vitamin from the food would lead to a relative deficiency. However, there is no evidence that the absorption of vitamin B_{12} should be impaired in healthy pregnant women. Moreover, Ross and Mollin (14) found the total amount of vitamin B_{12} stored in the liver of newborn infants to be small in comparison with the stores of the adult liver.

The highest values of the serum B_{12} were encountered in the age group 1 to 19 years. The reason for these high serum levels of the vitamin is not apparent. There was no evidence of a decrease of the serum B_{12} concentrations from 20 to 80 years of age which is in agreement with the results reported by Kristensen (8) but in contrast to those published by Mollin and Ross (10), Boger *et al.* (2) and Chow *et al.* (3). Possibly, these differences depend on the manner of selecting the material. The difficulties of finding "normal" old subjects are well known.

The Use of Serum B_{12} Assay in the Diagnosis of Vitamin B_{12} Deficiency

Deficiency of vitamin B_{12} usually gives rise to changes in the bone marrow and the blood picture, which are characteristic of pernicious

anaemia. In some patients, however, glossitis or neurological disease may precede the development of anaemia by various lengths of time. The difficulties of recognizing the true nature of the disease in such cases are well known. The same holds true for patients with a combined deficiency of vitamin B_{12} and iron. It seemed reasonable that serum B_{12} assay should be of value in the diagnosis of these disorders.

At first an attempt was made to define normal and subnormal serum B_{12} values by comparing a group of healthy subjects (156 haematologically normal subjects over 20 years of age) with a group of patients with undoubted vitamin B_{12} deficiency (182 cases of pernicious anaemia in relapse). Based on the results the following classification was adopted: Values of 100 $\mu\mu$g or less were termed subnormal, those between 100 and 150 border-line, and those between 150 and 1200 $\mu\mu$g per ml normal serum B_{12} concentrations. The frequency distribution of the serum B_{12} values reveals a clear difference between the two groups with only a small degree of overlapping of the results.

Following this a number of patients with various haematological and neurological disorders was studied. The patients had been admitted to different hospitals in Sweden and samples of blood were sent to this laboratory for analysis, because deficiency of vitamin B_{12} was suspected. As a rule, the samples were sent on the first few days after admission. With the aid of more extensive clinical investigations, supplemented in many cases by a therapeutic test with vitamin B_{12}, a definite diagnosis was established in most cases.

The results showed that 45 non-anaemic subjects with histamine fast achlorhydria had normal levels of serum B_{12} except for one case who had a border-line value. 145 patients had anaemia of varying aetiology: iron deficiency, chronic infections, cancer of the stomach, other malignant tumours and refractory anaemia. Three of the patients showed border-line serum B_{12} levels and 141, normal, or occasionally, slightly elevated values. In one patient, a 77-year-old man with cancer of the prostate and widespread, skeletal metastases, an extremely high serum B_{12} level (16,000 $\mu\mu$g per ml) was noted. Grossowicz *et al.* have shown that malignant growths in the liver are often associated with much elevated serum B_{12} concentrations (4). It is possible that this patient had metastases in the liver too, but unfortunately this was not investigated.

Eighteen patients suffered from megaloblastic anaemia not classified as pernicious anaemia. Eleven of these cases had subnormal serum B_{12} levels and responded satisfactorily to treatment with vitamin B_{12}. Three had normal serum B_{12} levels and failed to respond to treatment with the vitamin. Two of these improved on folic acid; the third died before treatment with this substance was begun.

Four patients with normal serum B_{12} concentrations improved following treatment with massive doses of the vitamin. In one of these

the anaemia was associated with phenyl-hydantoin and primidone treatment as described by Gydell (5) and in the remaining 3 with pregnancy or puerperium as described in paper V. It seems doubtful whether these last 3 cases suffered from deficiency of vitamin B_{12}. No satisfactory explanation of a possible vitamin B_{12} deficiency could be found. All had free hydrochloric acid in the gastric juice and responded slowly or incompletely to massive doses of the vitamin. It may be that the vitamin had exerted its effect by other means than by correcting a state of deficiency.

Three patients with glossitis had normal blood counts but subnormal serum B_{12} levels (III). The glossitis disappeared after treatment with vitamin B_{12}. In one of the cases the radioactive vitamin B_{12} absorption test according to Schilling (15) showed deficiency of intrinsic factor as in patients with pernicious anaemia.

In paper IV 11 patients with subacute combined degeneration of the spinal cord are described. All had subnormal serum B_{12} concentrations but showed either a normal blood picture, or haematological changes which were uncharacteristic in one or several respects. The Schilling test, performed in two patients, revealed deficiency of intrinsic factor. A comparison between the results of different laboratory investigations showed the serum B_{12} assay to be the most reliable method in the diagnosis of the condition. In four cases it was the determining factor.

Oral Maintenance Treatment of Pernicious Anaemia with Vitamin B_{12} and Purified Intrinsic Factor

The assessment of the long-term effect of drugs in the treatment of pernicious anaemia has always been difficult. If the drug is inadequate much time may elapse before significant haematological changes appear. It has been demonstrated that no correlation existed between serum B_{12} levels and red cell counts in patients with untreated pernicious anaemia (III). Moreover, patients with glossitis or subacute combined degeneration of the spinal cord showed very low serum B_{12} values although no or slight anaemia was present. Therefore, it was reasonable to presume that deficiency of the vitamin could be detected earlier by serum B_{12} assay than by other haematological methods.

An investigation on the use of the method for this purpose was started in December 1953 with a combined preparation of vitamin B_{12} and purified hog intrinsic factor (Bifacton). Twenty-four patients with pernicious anaemia, of whom 4 were in relapse, were followed for 15–39 months. At each follow-up haemoglobin concentrations, red cell counts, and serum B_{12} levels were estimated. A detailed account of the results is published in paper VI.

The 4 patients in relapse responded satisfactorily on a daily dose of 2–4 tablets of Bifacton and the previously subnormal serum B_{12} concentrations increased to normal. During prolonged treatment, however, the serum B_{12} began to decrease in most patients. After varying lengths of time the levels became subnormal in 19 patients, though at that time there were no overt signs of vitamin B_{12} deficiency (bone marrow biopsies were not investigated). These results suggested that the treatment was inadequate, but in order to prove that the subnormal serum B_{12} levels indicated a real deficiency of vitamin B_{12}, the therapy was continued. Subsequently, 10 patients relapsed after 15–28 months of treatment (mean 21 months). Two of these cases showed mainly neurological symptoms. The relapses were invariably preceded by subnormal serum B_{12} values, the intervals varying from 4 to 22 months (mean 12 months). Consequently, the finding of abnormally low serum B_{12} concentrations implies that haematological or neurological relapse may develop during this type of continued treatment. The 9 patients who had subnormal serum B_{12} levels but continued with normal blood counts were therefore regarded as pre-relapse cases. Only 5 patients were normal with regard to both blood picture and serum B_{12} concentration 24–29 months after the commencement of treatment.

In view of these experiences it was concluded (*a*) that serial estimations of the serum B_{12} levels provide a reliable means of demonstrating deficiency of the vitamin before the appearance of clinical symptoms, and (*b*) that combined oral preparations of vitamin B_{12} and purified hog intrinsic factor cannot be relied on for maintenance treatment in pernicious anaemia.

Several researchers have reported similar results using other preparations of vitamin B_{12} and hog intrinsic factor concentrates (e.g. Bendogen (1), Cycoplex MCO (9, 16)). There was no evidence that the appearance of relapses in this series was caused by failure of the patients to take their tablets. Nor was there any evidence that the preparation had become inactive by change of manufacturing procedure or during storage. In continued tests it was shown that different batches of the preparation were effective in producing remission in untreated pernicious anaemia (17). Moreover, in Schilling tests the intrinsic factor part of Bifacton promoted a normal absorption of vitamin B_{12} in 10 patients with untreated pernicious anaemia (VII). The cause of the relapse could not be explained by the use of too low maintenance doses. Increasing the dosage from 1 to 4 tablets per day did not influence the serum B_{12} levels or prevent the relapses (VI). For instance, although two tablets per day had produced a satisfactory remission in two patients, clinical relapse could not be prevented later on by doubling this dose. The results indicate that the patients had become resistant to the preparation.

Some possible explanations of this phenomenon have been investi-

gated. The experiments and results are presented in paper VII. At first, the absorption of radioactive vitamin B_{12} was studied by the Schilling test. Five patients in relapse and 2 patients in pre-relapse after prolonged Bifacton treatment did not absorb the vitamin when administered with the intrinsic factor of Bifacton. This finding is in contrast to the results obtained in the 10 untreated patients mentioned above. Consequently, the reduced effect of the preparation was due to the development of blocked absorption of vitamin B_{12}. However, when the labelled vitamin was administered together with normal human gastric juice the absorption appeared normal. These results indicate that the resistant patients were able to absorb vitamin B_{12} from the gut. All patients in relapse showed a complete haematological response following parenteral vitamin B_{12} therapy, which demonstrates that they utilized the vitamin normally.

Judging from the literature, relapses seem to be uncommon in patients treated with combined preparations of total hog pylorus and beef liver. This effect is probably not due to the presence of folic acid. It therefore seemed reasonable to study the effect of crude preparations of intrinsic factor and vitamin B_{12} on patients resistant to the purified substances. Crude intrinsic factor (muscularis and mucosa from hog pylorus) combined with crystalline vitamin B_{12} failed to overcome the blocked absorption in three patients. "Crude" vitamin B_{12} in the form of dried liver combined with purified intrinsic factor was unsatisfactory in another patient. In this case, however, a crude stomach—liver preparation appeared to be effective. Prolonged treatment of this case and similar experiments on other resistant patients are necessary before definite conclusions can be drawn.

As mentioned above, the resistant patients failed to absorb the labelled vitamin B_{12} together with the intrinsic factor of Bifacton but showed a normal absorption when human gastric juice was used as the source of intrinsic factor activity. Thus, it might be possible that the heterologous intrinsic factor of Bifacton had induced the formation of inhibiting substances, e.g. antibodies, which reduced the absorption of the vitamin. If this were true, another purified intrinsic factor preparation from the same animal might be expected to be also ineffective in promoting absorption of the vitamin in the resistant patients. To test this hypothesis, Schilling tests using intrinsic factor from both Bifacton and Bendogen were performed in two patients, who had relapsed following prolonged treatment with Bifacton. Both patients proved to be resistant to Bifacton but showed a normal absorption of the vitamin given with the intrinsic factor of Bendogen. These findings promoted a study of the long-term effect of Bendogen in patients resistant to Bifacton. Seven patients, of whom 6 were in pre-relapse, were treated with Bendogen. The latter showed an initial response to Bendogen, as revealed by increasing serum B_{12} concentra-

tions, but the present results of prolonged treatment suggest that 3 of these cases have developed resistance to this preparation.

Berlin *et al.* (1) found that 22 out of 66 patients relapsed after protracted treatment with Bendogen. The effect of Bifacton was studied in 12 of these Bendogen-resistant patients. There was no effect in 11 cases.

Development of resistance may therefore depend on the method of manufacture of the preparations. The fact that the resistant patients absorb vitamin B_{12} when it is administered with human gastric juice does not prove that homologous intrinsic factor is necessary for normal absorption of the vitamin. The results may just as well indicate that natural intrinsic factor contains unknown factors which are lacking in the purified mucosal preparations. The effect of prolonged treatment of homologous intrinsic factor has not been studied.

Of course, there are other possible explanations of the phenomenon of resistance, e.g. changes of the bacterial flora in the upper intestine. However, as yet, no satisfactory explanation has been found.

Concluding Remarks

The early diagnosis of vitamin B_{12} deficiency is of great importance especially in patients with subacute combined degeneration of the cord. It is equally important, however, that the diagnosis is correct as it usually implies a life-long control and treatment for the patient.

The results presented above (III, IV) indicate that serum B_{12} assay is the most reliable method of demonstrating vitamin B_{12} deficiency known at present. The method was shown to discriminate satisfactorily between patients with haematological or neurological disease due to deficiency of vitamin B_{12} and those suffering from other disorders of the blood and nervous system. It must be pointed out, however, that levels of serum B_{12} obtained by this method are not absolutely dependable. In this investigation a serum B_{12} concentration of 100 $\mu\mu$g per ml or less was considered as proof of vitamin B_{12} deficiency, but 9 out of 182 patients (5 per cent) with untreated pernicious anaemia showed values above this level (III). On the other hand, serum B_{12} concentrations less than 100 $\mu\mu$g per ml were found only in two patients without clinical evidence of vitamin B_{12} deficiency; the one suffering from acute leukaemia and the other from polyneuritis of uncertain nature (III).

The serum B_{12} assay does not offer any advantages over other methods in patients with obvious signs of vitamin B_{12} deficiency, e.g. pronounced pernicious anaemia. On the other hand, the present results

point to the method as the only conclusive proof of deficiency of the vitamin in some patients with glossitis or unclear neurological disease and in patients on inadequate oral maintenance treatment.

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